NON-HUMAN TRANSGENIC ANIMALS ABLE TO EXPRESS ANTI-NGF ANTIBODIES

Technical Field

The present invention relates to non-human transgenic animals for an anti-NGF (Nerve Growth Factor) antibody able to mimic neurodegenerative syndromes, to be used as a model to study said pathologies and provide therapies therefor.

Background

The nerve Growth Factor (NGF) (Levi-Montalcini, 1952) is a pleiotropic neurotrophin playing a fundamental role in the neuronal differentiation both at central and peripheral nervous system. Accordingly NGF proves to be indispensable for the differentiation of the cholinergic neurons of the basal telencephalon, the sensitive neurons and those of the sympathetic nervous system.

NGF is also necessary in steps following the differentiation, as it is able to modulate the phenomena of cellular apoptosis (Davies, 1992), synthesis of cytoskeleton elements during the neuroaxon regeneration (Snider, 1989), synthesis of peptide and enzyme neurotransmitters (Eide et al., 1993), synapse rearrangement and neuronal proliferation (Schnell et al., 1994) and that therefore it plays a fundamental role also in neurodegenerative processes and neuronal ageing (Connor & Dragunow, 1998).

The activity mechanisms regulating such different activities are yet to be clarified: the pleiotropic activity of NGF and unavailability of adult animal model do not allow to relate its expression to an unique and definite phenotype or prefixed and recognisable function. The inactivation of NGF in adult animal models has been attempted by different approaches, among which immunoneutralisation by systemic delivery of anti-NGF neutralising antibodies (Levi-Montalcini et al., 1960) o gene "knockout" in transgenic mice (Crowley et al., 1994).

However in both cases the results were rather disappointing because the systemically delivered antibodies do not pass the blood-encephalon barrier and the neutralising effect of NGF in the other districts depends on too many additional factors difficult to be standardised, like anti-serum level and affinity, clearance rate or polyclonal antibody cross-reactivity with other neurotrophins, etc.

On the other hand the "knockout" approach in mice allowed to confirm the importance of said neurotrophin and not redundancy of the intracellular signalling system thereof, but failed to provide the expected adult model for the study of the NGF: indeed the $ngf^{-/-}$ transgenic mice die shortly afterwards the birth before an eventual development of degenerative pathologies. The phenotype of $ngf^{-/-}$ heterozygotic mice also proved not very useful to study the pathologies associated with the NGF unavailability in adult: the NGF level in the heterozigotic mouse is only 25 % lower than in the control, it presents only a phenotype with a faint cholinergic deficit, without apparent anomalies and in relation with human neurodegenerative pathologies.

However various experimental evidences suggest that NGF plays a key role in neurodegenerative syndromes (Connor & Dragunow, 1988). Senile dementia and Alzheimer's disease (AD) are neurodegenerative syndromes characterised by a progressive dementia. The Alzheimer's disease affects 5 % of 70 year old people and more than 30 % of about 80 year old people. Its incidence, in relation to the improvement of the life conditions and lengthening of the mean age, is destined to double in the next thirty years. The social costs for said pathology are very high. The Alzheimer's disease first affects the neurons of the cerebral cortex and is characterised both by clinical (for example, the progressive loss of cognitive capacities) and characteristic pathological symptoms (which can be diagnosed only post-mortem), as the formation of extracellular plaques of β-amyloid protein, neurofibrillar intracellular aggregates, consisting of hyperphosphorylated tau protein and "neuronal loss" (Goedert, 1993; Mandelkow and Mandelkow, 1993; Selkoe, 1994). In the central nervous system the cholinergic system is particularly affected, resulting in a

decrease of the acetylcholine synthesis by the cholinergic neurons of the basal telenchephalon and a selective loss of said neurons. The cholinergic neurons are dependent on NGF which acts through high (TrkA) and low affinity (p75) receptors. Further the Alzheimer's disease is associated to motorial disorders resulting from the loss of cognitive capacities and coordination as well as from pathologies peculiar of the skeletal muscle as amyloid deposition within the skeletal muscle cells (Fukuchi et al, 10998; Jin et al., 1998). It is interesting to point out that NGF further exerts a function on non neuronal cells, also due to the presence of above NGF receptors, p75 and TrkA, on muscle cells too and for the finding of different isoforms of the TrkA receptor apparently not expressed in the nervous system.

In spite of enormous investments up to now an early diagnosis and a suitable therapy for the AD are unavailable. The reason results, above all, from the unavailability of experimental cellular or animal models which mimic in a complete and accurate way the formation of the aberrant neuropathological structures. During the last years various transgenic models have been suggested in order to define the aetiopathogenesis of the Alzheimer's disease and select useful compounds for the therapy thereof. Starting from the observation of the histological, immunological, and molecular characteristics of the Alzheimer's disease, as the presence of deposits of β-amyloid protein in the central nervous system, particularly at level of the cerebral cortex and in the hippocampus, have been obtained transgenic animals wherein the wild-type β-amyloid precursor protein (βAPP) is expressed at levels higher than endogenous or is expressed in a mutated form wherein the mutations are found in the genetic forms of the disease (Hsiao, WO 97/87492 and Games, WO 96/40896). Other suggested transgenic models relate to animals wherein the transgene is *PRESENILINE*-1 or –2 (Citron et al., 1996; Strchler-Pierrat et al., 1997), alone or together with the amyloid precursor protein (APP) (Borchelt et al., 1997; Holcomb et al., 1998; Wong et al., 1999), tau

protein (Gotz et al., 1995; Brion et al., 1999) or βAPP protein C-99 fragment (Jin et al., 1998).

However up to now all the suggested models develop only some of the morphological, histological or molecular markers defined as characteristic for the diagnosis of the Alzheimer's disease and therefore do not represent complete models, effectively suitable to study and provide successful therapies and test drugs.

Description of the Invention

In the present invention a non-human transgenic animal able to express ubiquitously an anti-NGF neutralising antibody is described. Further is described the method of preparation of the transgenic animal and the use of this model of phenotypic knockout to evaluate the NGF pleiotropic effects. A further aspect of the invention relates to the use of the transgenic mouse as animal model for the neurodegenerative syndromes, associated or not to muscle pathologies, as the Alzheimer's disease and the use thereof for the selection of the drugs suitable to block the neuronal degeneration or loss of the muscle activity. A further aspect of the present invention relates to the use of NGF to correct muscle pathologies and cholinergic deficit at cerebral level and neurodegenerative pathologies as AD.

Detailed Description of the Invention

The present invention describes a non-human transgenic animal able to express an anti-NGF (Nerve Growth Factor) neutralising antibody. The antibody used (αD11) binds NGF in correspondence to an epitope responsible for the bond with its high affinity receptor, TrkA, thus blocking the binding and therefore it acts as a neutralising antibody. The epitope recognised by the αD11 antibody (comprising the 41-49 amino acids of NGF) corresponds to a not very conserved sequence in the neurotrophins family and therefore it is NGF specific. Alternatively antibodies able to block the intracellular "signalling" can be used. Alternatively the recombinant antibody can be in the Fab, Fv or single chain Fv form.

Surprisingly the authors of the invention found that transgenic mice for anti-NGF antibodies, which do not express appreciable levels of the antibody in the period immediately after the birth and express such an antibody at levels between 50 and 500 ng/ml in the adult period, develop a complex pathological picture whose characteristic features are:

1) muscle dystrophy, particularly at level of the rear limbs;

2) dilatation of the cerebral ventricles, symptom often used in clinics as an indication of neurodegenerative diseases, like the Alzheimer's disease (Luxenberg et al., 1987);

3) atrophy of the cerebral cortex sometimes associated with the complete disappearance of the hippocampus;

4) loss of neurons and/or apoptosis, symptom related to the Alzheimer's disease (Mizutani et al., 1990);

5) deposition in CNS of plaques of β-amyloid protein, at level of the *PARA-CINGULAR* cortex and *NEOSTRIATUM*;

6) deposition of plaques of β-amyloid protein in the skeletal muscle;

7) neurofibrillar tangles and dystrophic neurites;

8) cognitive deficits characterised by defects in the "working memory" and spatial orientation deficits;

9) cholinergic deficit;

10) hyperphosphorylation of the tau protein at cerebral level;

11) hyperphosphorylation of the tau protein in the muscle:

12) infiltration of inflammatory cells in the muscle;

13) modifications of the sympathetic innervation of the spleen and reduction of the splenocyte viability;

14) aggregation of the tau protein in the encephalon.

An aspect of the present invention relates to the anti-NGF transgenic animal as a model to study the pathologies induced by the NGF deprivation. The NGF deprivation at systemic level can result in various aetiologies, of autoimmune type too. At local level a lack or limited availability of NFG by target cells can result, for example, from traumatic event (denervation).

Surprisingly many characteristics of the transgenic animal model described in the present invention are completely assimilable to those presented at macroscopic, histological and molecular level by the Alzheimer patients. The present invention therefore provides a non-human transgenic animal model to study the Alzheimer's disease. The characteristics of this model are summarised in table 1 where they are compared with those of other transgenic animals suggested for said disease. As it is clear form the table, the transgenic animal of the present invention exhibits characteristics which, as a whole, have never been observed in up to now provided animal models (Hsiao et al. 1996 and WO 95/48792 and WO 97/48792; Citron et al., 1997; Borchelt et al., 1997; Holcomb et al., 1998; Wong et al., 1999; Gotz et al., 1995; Brion et al., 1999; Jin et al., 1998; Games et al., 1995; Irizzary et al. 1997). Table 1 follows.

Table 1

DEFICIT	Transgenic mouse for						
	APP[1,5,9,10]	PS-1[2]	APP[1,5,9,10] and PS-1[2]	Human mutated tau protein[6]	Human mutated tau protein[7]	βPP C-99 fragment[8]	Anti-NGF antibodies (anti-NGF mouse)
Encephalon size	-	-	-	-	-	-	++
Thickness reduction of the cerebral cortex	-	-	-	-	-	-	+++
Cerebellum atrophy	-	-	-	-	-	-	+++
Dilatation of cerebral ventricles	-	-	-	-	-	-	+++
Memory and orientation deficits	++	-	-	-	-	-	+++
Neuronal loss	-	-	-	-	-	-	++
Apoptosis	-	-	-	-	-	-	++
Amyloid plaques	++	++	+++	-	-	-	++
Tau hyperphosphorylation	-	-	-	++	++	-	++
Neurofibrillary tangles/ neuropil threads	++	-	-	+	+	-	+++
Activation of the microglia cells	-	-	-	-	+	-	++
Cholinergic deficit	+	-	+-	-	-	-	+++
ATROPHY OF THE SKELETAL MUSCLES	-	-	-	-	-	++	+++

DEFICIT	Transgenic mouse for						
	APP[1,5,9,10]	PS-1[2]	APP[1,5,9,10] and PS-1[2]	Human mutated tau protein[6]	Human mutated tau protein[7]	βPP C-99 fragment[8]	Anti-NGF antibodies (anti-NGF mouse)
Amyloid deposits in the skeletal muscles (Congo red staining)	-	-	-	-	-	++	++
Amyloid deposits in the skeletal muscle (anti-APP IH)	-	-	-	-	-	++	++
Hyperphosphorylated tau IN THE MUSCLE (IH)	-	-	-	-	-	-	+
Macrophage infiltration	-	-	-	-	-	+++	+++
Vacuolization of the muscle fibre	-	-	-	-	-	+++	++
Increase in the number of nuclei in central position (myofiber)	-	-	-	-	-	++	++

[1]Hsiao et al. 1996; [2]Citron et al., 1997; [3]Borchelt et al., 1997; [4]Holcomb et al., 1998; [5]Wong et al., 1999; [6]Gotz et al., 1995; [7]Brion et al., 1999; [8]Jin et al., 1998; [9]Games et al., 1995; [10]Irizzary et al. 1997

Thus it is clear that the transgenic animal of the present invention constitutes a much more complete animal model for the Alzheimr's disease than those up to now suggested. Indeed the deficits recognised in the anti-NGF mouse, resulting from the transgene expression, are at level of both central and peripheral nervous system, at cognitive level, at muscle level and further at morphological-functional level in the spleen wherein anomalies in the expression pattern of the immunoglobulins can be found. In this regard the ever-increasing experimental evidences indicating as central the role played by the immune system in the development of the Alzheimer's disease are very interesting (Kalaria, 1993).

The preparation of the transgenic animal is carried out by crossbreeding of two strains of parent transgenic mice, which are transgenic because of the heavy and the light chain of an anti-NGF antibody, respectively, and such a method of preparation is a further aspect of the present invention. The two strains of the parent transgenic animals are prepared by microinjection of plasmid DNA in ES cells or oocyte fecundated according to standard methods known by those skilled in the art.

The plasmid vectors containing the transcription units for the cDNA expression of both immunoglobulin chains are purified, for example by banding technique using a CsCl continuous gradient, then diluting by physiological saline. The vectors can be injected as such in the pro-nucleus of fecundated oocytes, can be made linear by enzymatic restriction in a unique site or the transcription units can be separated from the vector by enzymatic restriction, purified, for example by gel electrophoresis or ion exchange chromatography, and separately introduced in the pronuclei. Preferably murine oocyte is used, more preferably it is from B6SJl strain. The two immunoglobulin chains of the anti-NGF antibody can be chimeric, obtained by assembling the variable murine regions of an anti-NGF monoclonal antibody, like Mab αD11, having human constant regions of K light and γ1 heavy chains, as described in the present invention or derived as such from the specific cDNA of the secreting hybridoma. The expression of

the two antibody chains in the trangenic mouse is controlled by a strong or viral promoter, as CMV-IE (Cytomegalovirus Immediate Early), which is often expressed ubiquitously. Other ubiquitous promoters which can be used are RSV (Rous Sarcoma Virus) LTR or SV40 (SV40-IE) early gene promoters. According to a further embodiment of the invention, in order to modulate the expression of the correctly assembled and functional only in particular districts of the organism the two transgenes can be brought under the control of two different promoters. In order to express the functional antibody only in a particular district or control the same over the time, can be used however tissue-specific or inducible promoters which can be different for the two antibody chains.

The preparation of the antigenic animal carried out according to the method of the invention determines the expression of the functional antibody in the adult transgenic animal at levels about 2000 times higher than at the birth and this allows, on one hand, according to the method of the invention, to increase up to 80 % the efficiency in the production of viable transgenic brood for both antibody chains, and on the other hand to point out the phenotype resulting from the NGF activity only in the adult period, avoiding the neutralisation thereof during the neuronal differentiation.

The transgenic mice obtained according to the method of the invention are able to express, at different level and in any case at amounts in the range from 50 to 500 mg/ml of serum, the functional chimeric antibody consisting of both the correctly assembled and secreted chains and said transgenic mice do not produce a lethal phenotype during the first post-natal period, as opposed to the transgenic animal obtained by microinjection of the plasmids encoding for both the immunoglobulin chains in the same oocyte.

The experiments of the authors of the invention pointed out that the neurodegenerative pathology at the encephalon level is preceded by an early (2 month age) tau hyperphosphorylation and amyloid deposition in the back or lower limb skeletal muscles. It is therefore within the scope of the present

invention the use of the skeletal muscle monitoring for an early diagnosis of neurodegenerative diseases.

Further it is pointed out that the muscular system phenotype of the anti-NGF mouse can be reversed by NGF local administration. According to its further aspect, the invention therefore is directed to the use of NGF for the preparation of pharmaceutical compositions to be used for the therapy of muscular pathologies, as muscular dystrophy/atrophy. The administration of said neurotrophin can be carried out by different routes among which there are: intramuscular injection of NGF, for example recombinant NGF, dissolved in suitable physiological saline, or direct injection of plasmid or recombinant viral vectors, for example adenovirus, or by implant in the muscle of cells genetically engineered for the NGF secretion. The dose can depend on various variables as the specific activity of the protein, severity of the pathology to be treated, general conditions of the patient and in any case will be form 2 to 100 μg/kg of body weight.

Description of the Drawings

Figure 1. Molecular characterisation of the parent transgenic mice (A): Transcription units used for the production of the transgenic mouse: light chain (upper panel) and heavy chain (lower panel). CK and CH1-CH3, human constant regions of the light (K) and heavy (γ1) chains; variable regions of the light (VL) and heavy (VH) chains of the αD11 monoclonal antibody; Cytomegalovirus pCMV promoter. (B): Scheme of the crossbreeding carried out to generate the anti-NGF transgenic mouse expressing the anti-NGF functional antibody. VK-αD11 X VH-αD11 (VK: transgenic parent line for the light chain of the αD11 antibody; VH: transgenic parent line for the heavy chain of the αD11 antibody. (C): PCR analysis of the VK (upper panel) and VH (lower panel) transgenes. From the gel it is possible to see the analytical results obtained for 12 homozygotic mice cross-bred with non transgenic mice. (D): Dot blot analysis of four murine lines expressing the light or heavy chain of αD11 antibody (VK-αD11 or VH-αD11). (E): VH-αD11 (left) and VK-αD11 (right) levels measured in the heart

at P1 and P90 days by Phosphoimaging analysis normalised for β-actin mRNA (count average +/- SEM).

Figure 2: Expression of the functional anti-NGF antibody in neuronal cells (A): Expression of the VH chain in Purkinje cells from cerebellum of transgenic mouse expressing the heavy chain (C line: negative control). Value of scale bar = 38 μm. (B): Expression of VK (left) and VH (middle) in DRG (Dorsal Root Ganglia) of family 1 anti-NGF mice (transgenic for both chains). The co-expression of both chains in the same cell is shown in the right panel. Value of scale bar = 75 μm. (C): Levels of mouse αD11 recombinant antibody from 1 and 2 family anti-NGF mice at 1st and 90th day, as measured in the serum (left) and encephalon (right). The dotted line represents the detection value in the assay (0,1 ng/ml and 0,1 ng/mg, respectively). (D): Control transgenic mouse (transgenic for VH only, below) and 3 family anti-NGF mouse (above), at 17th day. The anti-NGF mouse is smaller than the control. (E): Body weight of anti-NGF (family 1 and family 2) (left) and control mice (right).

Figure 3. Histological and molecular markers of the anti-NGF mouse A-H: sections of basal telencephalon; I-L: hippocampus sections. A-B: ChAT staining. A. control mice; B: anti-NGF mice. C-D: cholinergic innervation towards the frontal cortex. CP: caudate/putamen; FC: frontal cortex; GCC: genus of callosum corpus. C: control mouse; D: anti-NGF mouse. E-F: TrkA staining. E: control mouse; F: anti-NGF mouse. G-H: p75 staining. G: control mouse. H: anti-NGF mouse. I-L: ChAT staining. I: control mouse; H(L?): anti-NGF mouse. M and N sections: Timm staining of hippocampus "mossy fibres". M: control mouse; N: anti-NGF mouse.

Figure 4: Dilatation of cerebral ventricles Coronal sections of mouse encephalon stained according to Nissl method. The "aged" (15-18 month-old) anti-NGF mice have lateral ventricles (VL) dilated in comparison to the control mice. It is observed atrophy of the septohippocampus (SHI) and lateral nuclei (LS) of the septum.

Figure 5: Atrophy of the cerebral cortex Coronal sections of mouse encephalon obtained at the basal telencephalon level. The frontal cortex in transgenic mice (B) is atrophic in comparison to the control mice (A). The white bar indicates the cortex thickness measured in the same anatomical site. (C) Histogram comparing the atrophy grade in the transgenic (B) with respect to the control mice (A).

Figure 6. Atrophy of the hippocampus In comparison to those of the transgenic mice (B) the hippocampi of control mice (A) are perfectly formed. (C) Histogram comparing the formation grade in the transgenic (B) with respect to the control mice (A).

Figure 7. Neuronal apoptosis Labelling of cortical apoptotic neurons obtained according to the TUNEL method. In the control mice (A) positive cells are not observed, while in the anti-NGF mice (B) various nuclei with DNA fragmentation are observed.

Figure 8. Phosphorylation of the tau protein The transgenic mice show a remarkable positivity for the N-terminus segment of the tau (B), not phosphorylated tau (D) and above all phosphorylated tau protein (F). In the control mice the labelling is absent (A, C, E). The labelling is localised mainly at level of the cerebral cortex (for example in E). The labelling for the N-terminus segment of the tau protein (B) is present in some hippocampus cells (arrows).

Figure 9. Deposition of β-amyloid protein plaques in the encephalon Both the anti-APP monoclonal and polyclonal antibody against the β-amyloid precursor protein show the presence of plaques in the *PARA-CINGULAR* cortex and *NEOSTRIATUM* in control mice (A) and in higher number and larger size in the anti-NGF transgenic mice (B). In both figures the arrows point to the β-amyloid plaques.

Figure 10 Western blot of encephalon extracts from anti-NGF and control mice The experiment was carried out using the anti-tubulin (A), phosphorylated AT8 anti-tau (B) and amyloid precursor anti-protein (C)

antibodies. The blots are representative of 3 experiments wherein at least 3 control and 3 transgenic mice were used for each analysed age.

Figure 11 Accumulation of tau protein The insoluble fraction of the tau protein is accumulated in the encephalon of 5 month-old anti-NGF mice. The amount of the protein in the extracts was determined using the anti-tubulin antibody (A). The tissues were extracted in a sequential way using RAB Hi-salt (B), RIPA (C) buffers and 70 % formic acid (D). The tau insoluble fraction represented by the fractions extracted with RIPA and formic acid is accumulated in the anti-NGF mice but not in the extracts from the control mice. The columns 1-2 and 3-5 columns relate to the control and anti-NGF mice, respectively.

Figure 12 Deposits of amyloid in the cerebral cortex of anti-NGF (a,a) and control mice (b) Several extracellular deposits observed in the cerebral cortex of the anti-NGF mice show a fibrillar nature. Scale bar in a, b = 75 μm; c = 25 μm.

Figure 13 Presence of stiff amyloid plaques Stiff amyloid plaques are observed in the white subcortical matter (A) and cerebral cortex (B) of 6 month-old anti-NGF mice. The plaque morphology in 15 month-old anti-NGF mice (C) is similar to that of the plaques observed in encephalon sections from Alzheimer patients (D). Scale bar in A = 150 μm; in B-D = 75 μm.

Figure 14 (a, b) Sections of the parietal and (c) *ENTORINAL* cortex of anti-NGF mice NFT antibody shows the presence of tangles (neurofibrillar tangles) in pyramidal cells (arrows) and in dystrophic neurites (arrow tip). d, in sections of control mice no labelling is observable. Scale bar = 25 μm.

Figure 15 Neuron labelling by anti-NTF200 anti-tangles antibodies NTF antibody labels neurons in encephalon sections from both anti-NGF mice (G) and Alzheimer affected patients (H). Scale bar: 50 μm.

Figure 16 Time progression of neuron labelling by anti-MAP2 antibodies In the 2 (a), 6 (C) and 15 (E) month-old control mice the labelling obtained using the anti-MAP2 antibody is spread in the dendrites lengthwise. In the anti-NGF mice a decrease in the number of labelled dendrites and re-distribution of the

protein within the dendrites are observed. Such a decrease is progressive and affects the encephalon cortex of the 2 (B), 6 (D) and 15 (F) month-old anti-NGF mice. Scale bar: 100 μm.

Figure 17 *SILVER* impregnation (E,F) The *SILVER* impregnation shows the presence of extracellular deposits (asterisks) associated to dystrophic neurites (arrows) and tangle like formations (arrow tip). Scale bar: 50 μm

Figure 18 ChAT staining Choline acetyl transferase staining (ChAT) of the basal proencephalon of anti-NGF transgenic (c) and control mice (d). Scale bar: 200 μm.

Figure 19 Tau protein labelling 1 month after the birth mAb AT8 labels all the neurons of both the ENTORINAL (A) and parietal cortex (B). In both the cortices the labelling decreases 1,5 months after the birth. 2 month after the birth the AT8 positive neurons are present only in the ENTORINAL cortex (D), while only few neurons are labelled in the parietal cortex (F). no neuron is labelled in the ENTORINAL cortex of control mice (E). 6 months after the birth all the neurons in both the ENTORINAL (G) and parietal cortex are AT8 positve. No positive neuron is observable in the cerebral cortex of same age control mice (I). Scale bar: 100 μm.

Figure 20. Tau protein labelling 15 months after the birth AT270 (A, B) and AT8 (C, D) antibodies labels the pyramidal cells of the CA1 region of the anti-NGF mouse hippocampus. In the control mice the two antibodies labels only [AT270 (B) and AT8 (D)] axons. Scale bar: 100 μm.

Figure 21 Tau protein labelling The labelling obtained using mAb AT180 in anti-NGF mice increases with the age. Cerebral cortex 1 (E), 6 (F) and 15 (G) months after the birth. The arrows point to the dystrophic neurons, the arrow tips detect non neuronal positive cells. Scale bar: 75 μm.

Figure 22 mAb AT270 labelling in the cerebral cortex of anti-NGF mice 1 (A), 1,5 (B) and 2 (C) months after the birth. No difference is observable compared to the control mice. 6 months after the birth in the anti-NGF mice labelling in the cortical neurons (D) is observed. The number of the labelled neurons increases in the anti-NGF mice 15 months after the birth. 6 and 15

months after the birth the control mice do not show positive neurons in the cerebral cortex (F). Scale bar: 100 μm.

Figure 23 Muscular atrophy: Cross-sections of muscles collected from control and anti-NGF mice. Staining: haematoxylin eosine. Rectus medial M. of control (A) and transgenic (B) mouse leg. Gastrocnemius M of control (C) and transgenic (D) mouse. Anterior tibial M. of control (E) and transgenic (F) mouse.

Figure 24 Amyloid deposition in the muscle. The Congo Red staining detects the presence of amyloid deposits in transgenic (B) compared to control (A) mouse muscles.

Figure 25 Amyloid deposition in the muscle The immunoreactivity against the β-amyloid protein in the muscles of "aged" control mice (A) is completely absent. In the anti-NGF mice (B) a labelling increase, revealed by the brown precipitate, at the level of the sarcolemma and cytoplasm of the myofibres is observed.

Figure 26 Phosphorylation of the tau protein in the muscle The immunoreactivity against the phosphoryilated tau protein in the muscles of "aged" control mice (A) is completely absent. In the anti-NGF mice a labelling increase, revealed by the brown precipitate, at the level of the sarcolemma and cytoplasm of the myofibres (B) is observed.

Figure 27 Macrophage infiltration in the muscle Longitudinal (A) and cross (B) sections of "aged" anti-NGF transgenic mouse muscles. Among the myofibres the presence of immune type cells, as macrophages (arrows) is observed.

Figure 28 Position of the nuclei in the myofibres of the anti-NGF mice In some myofibres of "aged" mice the presence of nuclei in the middle of cell (arrows) rather than in the periphery is observed.

Figure 29 Behavioural test for anti-NGF mice A) Nociceptive test for anti-NGF transgenic and control mice. B) "Open field" test. C) "Rotarod" test. D) "Spatial orientation" plots for anti-NGF (n = 10, filled circles) and control (n = 10, empty circles) mice in the radial labyrinth test. The vertical bars represent

the standard error. The number of entries needed to find all the four meals is plotted against the time. E) Test for the conservation of the acquired functions, carried out on 35th day after the starting of the learning step. F) Transfer test of the acquired notions, carried out after the conservation test.

Figure 30 Test for the discrimination of the objects The test for the discrimination of the objects shows a defect in the differentiation among the objects in the anti-NGF mice, * $P < 0,03$.

Example 1 Production of anti-NGF transgenic mice and molecular characterisation thereof

Transgene preparation

Recombinant chimeric antibody was obtained by assembling the sequences of DNA corresponding to the variable murine regions of the Mab αD11 anti-NGF monoclonal antibody (Ruberti et al., 1993) (Genebank, access numbers: L17077/NID g310168, heavy chain and L17078/g310169, light chain, respectively) with the DNA corresponding to the constant regions of the human light K and heavy γ1 chains . The transcription units corresponding to the chimeric light and heavy chains (figure 1A), containing at 5' the Cytomegalovirus precursor and at 3' the polyadenylation site of the bovine growth hormone (bGH), cloned into the expression vectors pcDNAI-NeoVKαD11HuCK and pcDNAI-NeoVHαD11HuCγ, respectively, were extracted using KpnI-ApaI and KpnI-XbaI restriction enzymes, respectively, purified and injected separately or in combination in the pro-nucleus of mouse B6SJL strain egg cells fecundated according to standard methods, for example see Allen et al., 1987. Two transgenic parents for the light (A and B family, low and high producer, respectively), two for the heavy (C and D family, low and high producer, respectively) and three for both (double transgenic) antibody chains were obtained, respectively. The latter parents, which express the antibody at a level of about 50 ng/ml, are unable to reproduce and therefore are unsuitable for the continuation of the study.

The molecular analysis of the transgenic parent mice (A, B, C and D families) was carried out by PCR (Figure 1C) or Dot Blot (Figure 1D) on genomic DNA

extracted from tail biopsies as described in Piccioli et al., 1995. Messenger RNA was extracted according to Chomcynzki and Sacchi method, 1987, at different times form the birth and was analysed by RNAse-protection.

Preparation of the anti-NGF mouse

In order to generate animals transgenic for the functional antibody, consisting of both chains, the two parents transgenic only for the light (parents A and B) or only for the heavy (parents C and D) chains were cross-bred in different combinations (Figure 1B). Only the cross-breeds of A with D and B with C parents, which result in 1 and 2 family of double transgenic heterozygotic mice, respectively, are fertile and generate viable animals with an over 80 % efficiency.

Characterisation of the anti-NGF mouse

The levels of the functional antibody of either light or heavy chains of the transgenic animals were measured by ELISA assays (Monlar et al., 1998), using a biotin labelled human anti-IgG secondary antibody, after 1:10 dilution of serum or encephalon homogenates (Piccioli et al., 1995) with PBS-2 % powder milk.

The levels of the anti-NGF chimeric antibody for 1 and 2 families, measured in the serum and encephalon tissue of adult animals (90 day-old) are higher than 100 ng/ml and 100 ng/mg, respectively. The 2 family values are about two times than 1 family. Whereas soon after the birth (1 day) the antibody levels are lower than the detection limit of the assay (0,1 ng/ml in the serum and 0,1 ng/ml in the tissues) (Figure 2C).

The mRNAs specific for the chimeric VH and VK chains are expressed in different tissues among which the encephalon, kidney, heart, muscle, liver and testicles. The mRNA levels of both chains in the adult (90 day old) are about six times higher than in the newborn animal (1 day) (Figure 1E).

Therefore it results that the expression increase (1-2000X) of the anti-NGF functional antibody observed in the heterozygotic animal (double transgenic) is only partially determined by the increase of the mRNA expression levels.

Organ sections of anti-NGF mice are therefore fixed by intracardiac perfusion of 4 % paraformaldehyde in PBS, collected on a slide, preincubated in 10 % foetal serum and 5 % BSA, then used to detect by immunohistochemistry the expression of different antigenes: particularly the co-expression of the light and heavy chains of the anti-NGF antibody were made detectable by anti-human light or heavy chain biotinylate (Amhersham), detected by HRP or AP-conjugated avidin-biotin(Elite Standard kits, Vector). The localisation at cerebral level is showed in figure 2A, while in figure 2B is revealed by immunohistochemistry that the two chains of the chimeric antibody are co-expressed frequently.

Example 2 NGF phenotype knockout in anti-NGF transgenic mouse

The characterisation of the anti-NGF mouse phenotype was carried out at different levels: macroscopic, histological and molecular. At macroscopic level, during the first 3-4 life weeks the anti-NGF transgenic mice do not show remarkable abnormalities, except an about 25 % decrease of body weight compared to corresponding control mice (Figure 2 D and E). Usually the experiments were carried out on anti-NGF transgenic animal of numerosity group n = 6 with anti-NGF antibody levels from 50 to 300 ng/ml; as controls transgenic mice were used only for the antibody heavy chain (VH) (parent C or D), therefore not expressing the functional antibody.

At histological and molecular level the following differences, compared to normal mice, were observed, district by district: 1) central and peripheral nervous system, 2) muscular system and 3) spleen.

1) Central and peripheral nervous system

IN THE BASAL TELENCEPHALON AN UP TO 57 % REDUCTION IN THE NUMBER OF ACETYLTRANSFERASE-POSITIVE NEURONS (CHAT) AND A REDUCTION OF THE EXPRESSION LEVEL WERE OBSERVED, WHILE IN THE HIPPOCAMPUS AN UP TO 70 % REDUCTION OF NEURONS IS OBSERVABLE. IN ADDITION THE CELLS APPEAR MORPHOLOGICALLY SMALLER. AS FOR THE PERIPHERAL NERVOUS SYSTEM THE UPPER CERVICAL GANGLIA ARE UP TO 45 % SMALLER THAN CONTROL; ALSO IN THIS CASE THE CONSTITUTING CELLS APPEAR MORPHOLOGICALLY SMALLER (FIGURE 3 A-

B). THE MORPHOLOGICAL AND HISTOLOGICAL ASPECT OF THE MOUSE ENCEPHALON EXPRESSING THE ANTI-NGF RECOMBINANT ANTIBODY WAS ANALYSED IN TRANSGENIC 15-18 MONTH-OLD MICE ("AGED" ANIMALS) IN COMBINATION WITH THE PRESENCE OF PHENOTYPIC LABELS OF NEURODEGENERATIVE DISEASES, AS FOLLOWING: "NEURONAL LOSS" AND APOPTOSIS, SYNTHESIS OF CHOLINE-ACETYLTRANSFERASE (CHAT) (FIGURE 3 A, B), DETERMINED BY IMMUNOHISTOCHEMISTRY WITH ANTI-CHAT ANTI-SERUM (CHEMICON), RATIO OF PHOSPHORYILATED TO NON PHOSPHORYLATED TAU PROTEIN (MEASURED BY IMMUNOHISTOCHEMISTRY OR WESTERN BLOT WITH SPECIFIC ANTISERA), PRESENCE OF β-AMYLOID PROTEIN AND PRECURSOR OF PHOSPHORYLATED AMYLOID PROTEIN, APP (AMYLOID PRECURSOR PROTEIN), (DETERMINED BY IMMUNOHISTOCHEMISTRY WITH SPECIFIC ANTISERA),. THE NUMEROSITY OF THE GROUPS USED FOR THE EXPERIMENTS, EXCEPT WHERE OTHERWISE INDICATED, WAS N = 6 TRANSGENIC ANTI-NGF WITH TRANSGENIC ANTI-NGF ANTIBODY LEVELS FROM 50 TO 300 NG/ML; AS CONTROL WERE USED MICE TRANSGENIC ONLY FOR THE ANTIBODY HEAVY CHAIN (VH) (PARENT C AND D), THEREFORE NOT EXPRESSING THE FUNCTIONAL ANTIBODY.

The obtained results can be summarised in the following points:

(a) Dilatation of the cerebral ventricles (Figure 4). The severity of the ventricular dilatation is associated to a remarkable atrophy of the cerebral cortex (Figure 5) and hippocampus (Figure 6).

(b) Neurodegeneration and neuronal loss. It is possible to visualise apoptotic cells in more severely damaged mice at cerebral level as pointed out by TUNEL method (Figure 7). Apoptotic phenomena indicate a progressive cell die.

(c) Decrease of the choline-acetyltransferase (ChAT) synthesis, (Figure 3 A-B and I-L), particularly in the basal telencephalon. Namely no neurons positive for this label in the nucleus of the medial septum is observed in analysed animals. The expression, in comparison to that observed in younger (2-3 month-old) mice, is decreased. A lower expression is also observed for the two NGF-receptors, TrkA (Figure 3 E-F) and p75, in the

basal telencephalon (Figure 3 G-H). Particularly the decrease for the TrkA-positive is more remarkable than for p75-positive cells.

(d) Increase of the tau protein phosphorylation (Figure 8). Using antibodies specific for tau protein N-terminus segment [Alz-50 (Wolozin et al., 1986)] (Figure 8A and 8B), against the not phosphorylated tau protein [anti-Tau 1 (Grundke-Ipbal et al., 1986)] (Figure 8C and 8D) and against the same epitope of the phosphorylated tau protein [mAB AT-8 (Greenberg and Davies, 1990)] (Figure 8E and 8F), by immunohistochemistry a remarkable generalised increase of the tau synthesis, mainly of the phosphorylated component thereof, was detected. The used antibodies labels cortical neurons which present a modified morphology, evidencing the presence of "neuropil threads", "ghosts" and "tangles". The labelling against the phosphorylated form of tau protein evidenced a remarkable increase of this protein also in the microglia cells which are activated in neurodegenerative processes. Antibodies against the β-amyloid protein and amyloid precursor protein [APP (Glenner and Wong, 1984)] evidence the presence of several plaques in both the *PARACINGULAR* cortex and *NEOSTRIATUM* (Figure 9A and 9B). In a further experiment the progressive increase of the hyperphosphorylated tau content in the encephalon of the anti-NGF mice was evaluated by biochemical analysis (Western blot analysis). The results were normalised for the total protein content using an antibody against tubulin (Figure 10A). The western blot analysis carried out using the antibodies against hyperphosphorylated tau (PHF-1 and AT-8) evidenced that an increase of the phosphorylated tau content is present in 2 month-old animals and the tau content reaches a plateau 6 months after the birth (Figure 10B). The biochemical analysis of the amyloid precursor protein evidenced that the content of this protein increases from 6 months after the birth (Figure 10C). Furthermore 15 months after the birth two bands, corresponding to 120 kDa and 25 kDa, respectively, are observed (Figure 10C).

The presence of insoluble aggregates of tau protein was evaluated in 15 month-old anti-NGF mice. The encephalon was extracted sequentially using buffers with different extraction activity. The experiments evidenced that in the anti-NGF mice most hyperphosphorylated tau protein is insoluble (Figure 11).

Therefore the modification at the protein level of the tau cytoskeleton precedes the modifications observable at the amyloid protein level. Further the experiments evidence the presence of insoluble tau, which can be part of that component forming PHFs (paired helical filaments) which constitute the intracellular tangles and extracellular deposits in the Alzheimer disease. The results show a modified processing of the amyloid protein too.

The presence of amyloid plaques was detected using an antibody against the amyloid precursor protein. The experiments were carried out using both immunohistochemical and Western blot techniques (see above). The results showed that, 15 months after the birth, amyloid plaques are present in both the cerebral cortex and hippocampus of anti-NGF mice (Figure 12). These plaques cover a significant part of the *ENTORINAL* cortex surface, the percentage values being 21 % of the surface compared to 0,5 % in the control mice. In other regions of the cerebral cortex the percentage of the surface covered by amyloid plaques is 10 % and 0,1 % in the anti-NGF mice and control mice respectively. The values are 4 % and 0,1 % in the hippocampus of the anti-NGF and control mice, respectively.

The plaque distribution and morphology thereof in the anti-NGF mice are entirely comparable to those observed in sections of patients affected by Alzheimer's disease (Figure 13). From above data it can be concluded that in the anti-NGF mice an high extracellular deposition of amyloid as plaque aggregates similar, as for morphology and distribution, to those observed in human encephalon sections of patients affected by Alzheimer's disease.

(e) Presence of intracellular tangles in neurons of anti-NGF mice. The presence of intracellular tangles in encephalon sections of anti-NGF mice was showed using a mAB NFT200 antibody able to detect tangles in

encephalon sections of Alzheimer affected patients. mAB NFT200 labelled many neurons distributed throughout the encephalon of anti-NGF mice (Figure 14a-c) but not in control animals (Figure 14d). The antibody detected the presence of intracellular inclusion in dystrophic neurites too. In Figure 15 it is possible to compare the distribution of tangles in anti-NGF mice and in encephalon sections of Alzheimer patients.

The mAB NFT200 antibody reveals, in the encephalon of anti-NGF mice, aggregates similar to those observed in sections of human encephalon. This feature, indispensable to confirm the diagnosis of the Alzheimer disease in humans, was never detected up to now in other animal models partially reproducing this pathology.

(f) Modifications of the distribution of the protein associated to MAP-2 microtubules. The protein associated to the microtubules (MAP-2) is part of the multiplicity of the proteins forming the cytoskeleton of neurons. The modifications of said protein were detected using the anti-MAP-2 antibody. 1 and 1,5 months after the birth the observed distributions of the MAP-2 protein in the cortex neurons of anti-NGF transgenic and control mice were similar. 2 months after the birth in the control mice the MAP-2 labelling is distributed throughout the dendrites lenghtwise (Figure 16A). At this age in the anti-NGF mice a decrease in the number of labelled dendrites and a labelling re-distribution in the dendrite lengthwise are observed (Figure 16B). 6 and 15 months after the birth the number of dendrites is still decreasing in the anti-NGF mice. In the dendrites of these animals a clear labelling re-distributrion is also observed, which is localised in the proximal zone of the dendrites (Figure 16D,F). In the same age control mice the MAP-2 labelling is still distributed throughout the dendrites lengthwise (Figure 16C,E).

From these results it can be deduced that the NGF deprivation determines a modification in the distribution of the cytoskeleton proteins of the cortical neurons. This modification could be part of the neurodegenerative phenomena leading to the occurrence of the Alzheimer disease.

(g) Silver impregnation of anti-NGF mice encephalons. For these experiments a silver impregnation technique (Bielschowsky metohod), previously used to detect extracellular neurite plaques and tangles in encephalon sections of Alzheimer patients, was used.

In the anti-NGF-mice this technique allowed to detect the co-existence of dystrophic neurites and extracellular fibrous material in the form of plaques (Figure 17E,F). These aggregates are clear in 6 and 15 month old mice.

The silver impregnation is an histological technique which allowed, independently from immunohistochemical techniques, to detect the presence of plaques consisting of extra-cell deposited material and dystrophic neurites. In addition this technique allowed to detect the co-existence of these two modifications. The attempts to detect these modifications in other animal models for the Alzheimer disease failed.

(h) Time progress of the neuropathology in anti-NGF mice. Experiments to evaluate the occurrence of modifications in the different phenotype markers were carried out. This time progress is summarised below and in table 2:

1. The decrease of cholin-acetyltransferase(ChAT)-positive neurons, previously described (Ruberti et al., 2000), continues 2 months after the birth and reaches a plateau 6 months after the birth whereupon a 90 % reduction in the number of positive neurons in the medial septum (Figure 18C,D) is observed.

2. The determination of the somadendritic distribution of the tau protein in hyperphosphorylated form was carried out by different antibodies and, in the anti-NGF mice, showed as follow:

- 2 months after the birth only the entorinal cortex presents modification detected by mAB AT8 (Figure 19),
- these modifications extend to other regions of the cerebral cortex and hippocampus (Figure 19 and Figure 20) from 6 months after the birth,
- these modifications are detected also by other antibodies, different from AT8, i.e. AT180 and AT270 mABs.

3. The AT8 antibody, used together with extraction techniques, shows that the most tau protein extracted from the anti-NGF mice encephalon is insoluble.

4. The cytoskeleton modifications concern not only the protein but also MAP-2 protein and start 2 months after the birth.

5. The tangle-like inclusions are present only 15 months after the birth, whereas the dystrophic neurites are detected already 6 months after the birth.

6. The DNA fragmentation is observed only 15 months after the birth. In conclusion the anti-NGF mice present a time progress of the neurodegeneration starting from the cholinergic deficit and modification of some cytoskeleton proteins. The spatial progress of the pathology is similar to what observed in encephalon form Alzheimer patients.

Table 2 follows.

Table 2

		Age (months)				
phenotypic markers	cerebral zones	1	1,5	2	6	15
ChAT decrease		-	-	+	++	++
Presence of hyperposphorylated tau protein in somadendritic compartment						
AT180	Entorinal cortex	-	-	-	+	++
	Parietal cortex	-	-	-	+	++
	Occipital cortex	-	-	-	+	++
	Hippocampus	-	-	-	-	-
AT270	Entorinal cortex	-	-	-	+	++
	Parietal cortex	-	-	-	+	++
	Occipital cortex	-	-	-	+	++
	Hippocampus	-	-	-	-	+
AT8	Entorinal cortex	-	-	+	++	++
	Parietal cortex	-	-	-	+	++

		Occipital cortex	-	-	-	+	++
		Hippocampus	-	-	-	+	++
	Insoluble tau		nd	nd	nd	nd	++
	MAP-2 abnormal subcellular localization		-	-	+	++	++
	Neurofibrillar tangles		-	-	-	-	++
	Amyloid plaques		-	-	-	+	++
	Inclusions detected by silver impregnation		-	-	-	+	++
	Dystrophic neurites*	Entorinal cortex	-	-	-	++	++
		Parietal cortex	-	-	-	+	++
		Occipital cortex	-	-	-	+	++
		Hippocampus	-	-	-	-	-
	DNA fragmentation	Cerebral cortex	-	-	-	-	+
		Basal proencephalon	-	-	-	-	-

+ means a qualitative measure of each phenotypic marker, ND, not determined.

* as detected by silver and immunohistochemical impregnation by hyperphosphorylated anti-tau antibodies and "tangles".

Therefore the analysis of these mice showed that the neurogenerative pathology at the encephalon level is preceded by an early (2 months after the birth) tau hyperphosphorylation and amyloid deposition in the back or lower limb skeletal muscles. It is to be pointed out that the association of Alzheimer disease with inclusion body myositis in humans is already known.

In summary the transgenic mice expressing the anti-NGF antibody resemble at the level of the Central and Peripheral Nervous System many pathological modifications typical in neurodegenerative diseases, particularly Alzheimer disease.

2) Muscular system

Mice evaluated (n = 15), from 45 to 60 days after the birth, at a macroscopic level stagger, due to an abnormal position of the rear legs and support of toe tips and often present backbone scoliosis. The anatomical analysis shows a reduction of the back longitudinal skeletal muscles, flexor and adductor of the rear limbs, feature not observable in other muscles, for example in the corresponding muscles of the front limbs. Some deficits were better characterised and detailed as follow:

a) muscular dystrophy, characterised from the morphological and histological point of view. The atrophy of the muscular fibres is present, in all the considered animals (n = 15), for the muscles which allow the movements of the backbone and aid the stability of the connections of each other vertebra (longest muscle for the backbone and inter-vertebral muscles, respectively). Further in all the animals the reduction of the diameter of the muscular fibres (up to 50 %) is observable in the 70 % of the fibres: in the adductor (leg medial rectus, large and small adductor), leg flexor (outer, medial and inner ischiotibial) and metatarsus extensor (gastrocnemius and soleus muscles). On the contrary the atrophy is not present at the level of the metatarsus flexor muscles (front tibial and phalanx extensor muscles) and it is less evident in the front limb extensor muscles (brachial triceps muscle). All these differences are showed in Figure 23. Further every dystrophic muscular fibre show also a remarkable vacuolization (Figure 23B-E) and a more intense staining by haematoxylin/eosine.

b) scoliosis, in some animals (n<6), in some cases associated with an incomplete development of the vertebral bodies.

c) muscular atrophy, typified at molecular level as follow:

c.1) re-expression of the low affinity NGF receptor (p75). It is particularly clear in some muscular cells exhibiting modifications in the distribution of nicotine receptors at the level of the neuromuscular junctions.

c.2) decrease in the number of the nervous peptidergic endings at the level of the neuromuscular junctions. This decrease was detected by

antibodies against the calcitonin gene-related peptide [CGRP (Gibson et al., 1984)].

c.3) absence of the aggregation of the acetylcholine receptors in the plasmatic membrane of the muscular cells and detected by the irreversible binding of alfa-bungarotoxin (Changeux, 1991), caused by the failed innervation of the muscular fibre. The distribution of the muscular cells exhibiting such a modification gives the muscles of the transgenic mice a characteristic mosaic pattern.

c.4) increase of the dystrophin immunoreactivity detectable in the above described cells exhibiting molecular modifications by immunohistochemistry using D-8043 antibody (Sigma). The dystrophin is a protein of the cytoskeleton muscular cell involved in the contraction and aggregation of the cholinergic receptors. It is already known that an increase in the dystrophin synthesis occurs concurrently with the muscle denervation.

c.5) ATPase modified metabolism due to the lack of the nerve trophic effect.

c.6) remarkable deposition of amyloid substance, detected by a characteristic ring cytoplasmic staining by Congo Red (Figure 24A-B). The presence of amyloid and particularly β-amyloid was detected also by immunohistochemistry against the β-amyloid precursor protein (Figure 25A-B) in aged mice.

c.7) phosphorylated tau protein in the muscles of the aged mice (age from 15 to 18 months). Figure 26A-B.

c.8) presence of various muscular fibres with nuclei located in the middle rather than below the sarcolemma in aged anti-NGF mice (Figure 28). Histological assays detects an infiltration of immune type cells, probably macrophages, among the muscular fibres (Figure 27A-B).

The presence of deposits of the β-amyloid and hyperphosphorylated tau protein and in addition nuclei located in the middle region and macrophages infiltration, is related to what observed in inclusion body myositis (IBM), a pathology strictly correlated with the Alzheimer disease.

3) Spleen

At anatomical level the localisation of the sympathetic innervation is distributed in the germinal centre and marginal zone, rather than in the proximity of the central artery, as in the control mice. The recover of the viable splenocytes is reduced by one order of magnitude (2-3 x 10^6 vs. 2-3 x 10^7 of the controls) in the anti-NGF transgenic mice, as observed by flow cytometry. Functionally it can be observed a reduction of the number of the IgG positive lymphocytes and a fair increase of the IgD positive lymphocytes, as measured after incubation (30', 4°C) of the splenocytes with FITC anti-IgG (Sigma), IgM, IgA, IgD mouse (Pharmingen) labelled primary antibodies and analysis by Coulter Epics Elite Esp Flow Cytometer at 488 nm. Furthermore in the red pulp can be detected DNA fragmentation indicating apoptosis according to the reduced recover of viable splenocytes.

Example 3 Analysis of the behaviour of the ant-NGF transgenic mice

The analysis was carried out on 12-18 month old animals (n = 6), selecting animals without evident gait anomalies. The following anomalies, resumed in Figure 29, with respect to the control animals were detected:

- increase of the latency time for the heat sensitivity, changed from 3" for the control mice to 16" for the anti-NGF mice, as measured according to the hot plate nociceptive assay, already described in Eddy et al., 1953;

- spatial orientation: the anti-NGF mice exhibit a higher number of errors during the working memory learning over first three days, in fact the learning plots are significantly different (two way RMANOVA test, $p<0,05$), however the final learning level is not different from that of the control mice, as measured by the radial labyrinth test, carried out as follow: the animals were located in 8 arm radial labyrinth and free to feed themselves for 5' and familiarise with the labyrinth over two days. For the test the same four arms were filled with food every day; at the beginning of each test the mice were left at the centre of the labyrinth, free to explore it: the test was terminate if the food was finished or 25 entries were observed into the arms of the labyrinth: the tests were repeated twice a day over 14 days, made mistakes

(short and long term memory mistakes) and taken times being measured. The starting and final learning levels were evaluated using the average of the mistakes made over the first and last three days.

- ability in maintaining the acquired notions. The anti-NGF mice do not maintain the acquired notions at 31st day from the learning step, as measured by the same radial labyrinth test. The learning plots were compared with the two way ANOVA test (treatment x time) and the significance of the differences evaluated by T-test.

- deficit in the ability of learning transfer into other situation, as measured by the radial labyrinth test, using food filled exits different from those used in the learning step. The anti-NGF mice exhibit a clear learning deficit ($p < 0,01$ in two way RMANOVA test) in comparison to the control mice, also after 5 learning days. The differences resulted mainly from a higher number of short term memory errors (T-test, $p<0,006$).

- short term memory test (object discrimination test). According to this test the mice explored 2 white cubes over 10 minutes. Then one cube was coated with white and black chess painted paper. One hour after the end of the first trial the mice were allowed to come again in contact with the cubes and explore them over additional 10 minutes. The anti-NGFtransgenic mice were not able to distinguish between the two cubes coated with differently coloured papers (Figure 30). Therefore the anti-NGF mice show a decrease in the short term memory, not being able to memorise and distinguish between the two differently coloured cubes.

Example 4 Reversibility of the muscular dystrophy in anti-NGF mice by NGF local administration

All the experiments were carried out on 45 day old mice, when the serum level of the anti-NGF antibody is reaching the highest level (observed 60 days after the birth). The NGF was administered locally by different methods: (a) by intramuscular injection of NGF, (b) by a viral recombinant vector (adenovirus) encoding for NGF cDNA or (c) by the implant of NGF secreting fibroblasts. All the administration ways included the injection or

implant in the gastrocnemius muscle, one of the skeletal muscles affected by muscular dystrophy. The injections and implants were carried out on the right leg, while the gastrocnemius muscle of the left leg was used as control. A) NGF was injected as pellet, consisting of diazocellulose mixed NGF in borate buffer at pH 8,0 for 72 hours and following neutralisation by glycine saturated solution (Hendry, 1982). This method allows the exact localisation and slow release of this neurotrophin. Different NGF concentrations were used, comprised in the range from 100 μm and 2 mg for each animal. For the administration of cDNA according the method b), 10 μl of the adenoviral vector solution corresponding to 10^7 pfu/ml were injected in the gastrocnemius muscle. In the control animals a recombinant adenovirus containing Escherichia coli Lac Z reporter gene was injected. Both in this and in fibroblast injection experiment (see later) the NGF production was constant at least over one month, allowing the phenotype reversibility to be observed.

According to method c) fibroblasts genetically modified to secrete NGF by infection with a retroviral vector encoding for cDNA of this neurotrophin, according to the method described by Gage et al. (1990), were implanted in the muscle. This allowed to obtain an in situ NGF production equal to 100 ng/10^6 cells/day. The fibroblasts were injected after re-suspension in sterile physiological saline at a 2 x 10^5 cells/μl concentration. The injection volume was 10 μl/animal.

To verify the effect of the NGF administration the animals were sacrificed 7, 15 and 30 days after the injection. The injected and contralateral muscles were collected and analysed by histological and immunohistochemical techniques to verify the attenuation of the dystrophy and the restoration of usual innervation. Thus it was verified that in all the injected animals the values of the muscular fibre diameter were again similar to those of the control animals. Furthermore their morphology and cholinergic and peptidergic innervation re-assumed an usual appearance.

As to the restoration of the cholinergic phenotype in the basal telencephalon two different approaches were used. In a first set of

experiments after the anaesthesia the equipment of NGF releasing minipumps was used. A rubber capillary tube was inserted in the lateral ventricle and then connected by means of an osmotic minipump to a "pocket" of the subcutaneous layer. This pump was filled by NGF (30 – 100 μg) diluted with Ringer-Locke physiological saline. Another group of animals was treated by an implant of fibroblasts genetically modified to secrete NGF. The fibroblasts were injected in the lateral ventricle at a 2 x 10^5 cells/μl concentration. The injection volume was 1,5 μl/animal. This second method allowed to verify, by E.L.I.S.A., the constancy of the NGF production over 4 weeks after the implantation. The analysis of the cholineacetyl transferase expression (ChAT) in the nuclei of the basal telencephalon and the analysis of the behaviour of these mice allowed the phenotype reversibility to be observed in the anti-NGF mice at the level of the cholinergic system.

As a whole these results confirm that the NGF administration is able to correct the muscular and cholinergic deficit observed in the anti-NGF mice.

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